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In Vitro Suppression of K65R Reverse Transcriptase-Mediated Tenofovir- and Adefovir-5′-Diphosphate Resistance Conferred by the Boranophosphonate Derivatives[▽]

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9-[2-(Boranophosphonomethoxy)ethyl]adenine diphosphate (BH_3-PMEApp) and (*R*)-9-[2-(boranophosphonomethoxy)propyl]adenine diphosphate (BH_3-PMPApp), described here, represent the first nucleoside phosphonates modified on their α-phosphates that act as efficient substrates for the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). These analogues were synthesized and evaluated for their in vitro activity against wild-type (WT), K65R, and R72A RTs. BH_3-PMEApp and BH_3-PMPApp exhibit the same inhibition properties as their nonborane analogues on WT RT. However, K65R RT was found hypersensitive to BH_3-PMEApp and as sensitive as WT RT to BH_3-PMPApp. Moreover, the presence of the borane group restores incorporation of the analogue by R72A HIV RT, the latter being nearly inactive with regular nucleotides. The BH_3-mediated suppression of HIV-1 RT resistance, formerly described with nucleoside 5′-(α-*p*-borano)-triphosphate analogues, is thus also conserved at the phosphonate level. The present results show that an α-phosphate modification is also possible and interesting for phosphonate analogues, a result that might find application in the search for a means to control HIV RT-mediated drug resistance.

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) plays a key role in the viral life cycle and is therefore an important target for antiretroviral drugs such as nucleoside and nucleotide RT inhibitors (NRTIs). After intracellular phosphorylation, these analogues are selectively incorporated into the viral DNA, leading to the observed antiviral effect through premature termination of viral DNA synthesis. Although effective at reducing viral loads in HIV-1-infected patients, NRTI use has resulted in the development of mutations in RT that contribute to HIV-1 drug resistance.

Tenofovir disoproxil fumarate, the oral prodrug of tenofovir [(*R*)-9-(2-phosphonomethoxypropyl)adenine {(*R*)-PMPA}] (6) was approved for the treatment of HIV-1 infection in 2001. Tenofovir is an acyclic nucleoside phosphonate (ANP) analogue of dAMP (2′-deoxyadenosine 5′-monophosphate) that requires two phosphorylation steps by cellular kinases to become the active metabolite tenofovir diphosphate (PMPApp) and act as a DNA chain terminator. There is considerable interest in phosphonate mimics because of their long intracellular half-lives (10) and their capacity to circumvent the rate-limiting first phosphorylation step (19). The lysine-to-arginine substitution at residue 65 (K65R) in HIV-1 RT is among the most relevant resistance mutations (25) selected by tenofovir in vitro and in vivo. This mutation is also selected by other nucleosides (4, 12, 23, 25) and adefovir [9-(2-phosphonylmethoxyethyl)adenine (PMEA)] (11), conferring reduced susceptibilities to these drugs. Although tenofovir does not select for *t*hymidine-*a*ssociated *m*utations (TAMs), the presence of specific combinations of TAMs resulted in reduced susceptibility to tenofovir in vivo (17). Several mechanisms are known to contribute to decreased drug susceptibility. One mechanism explaining the decreased susceptibility of K65R RT to tenofovir is the decreased ability of the K65R RT to bind to and incorporate tenofovir diphosphate into proviral DNA, as measured by a 3.5-fold increase in K_i (25). Indeed, the K65R substitution affects the interaction between the enzyme and the triphosphate moieties of NRTIs and might therefore alter the analogue binding specificity or the phosphodiester bond formation kinetics (26).

Based on the knowledge of the mechanisms of resistance to tenofovir (2, 21, 24, 26) and adefovir diphosphate (PMEApp) (11), we wanted to explore the drug susceptibility in vitro of (*R*)-9-[2-(boranophosphonomethoxy)propyl]adenine diphosphate (BH_3-PMPApp) and PMEApp analogues, as the "borane" modification has been reported to overcome resistance. Indeed, mutant RT resistant to the cognate nonborane inhibitor recovers sensitivity upon the introduction of an α-boranophosphate group into the triphosphate form of clinically relevant compounds, such as zidovudine, stavudine, and dideoxyadenosine (1, 7, 8, 18). The presence of the borane group does not influence the binding affinity of the analogue for the RT active site but specifically either provides or restores a high rate of incorporation of the analogue by wild-type (WT) or mutant HIV-1 RTs, respectively (1, 7). The focus of the present study is thus to investigate in vitro the effect on susceptibilities of HIV-1 WT and mutant RTs to the borane modification introduced at the α-positions of acyclic phosphonate diphosphates. We have explored the in vitro activity of α-boranophosphonate diphosphates BH_3-PMEApp and BH_3-

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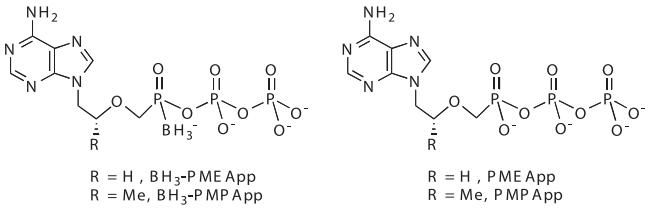

FIG. 1. Chemical structures of α-boranophosphonate diphosphates BH_3-PMEApp and BH_3-PMPApp and their non-boranophosphonate diphosphates, PMEApp and PMPApp.

PMPApp (Fig. 1) against WT RT and against both K65R and R72A mutants. The arginine 72 side chain contacts the α-phosphate of the incoming nucleotide. Consequently, R72A RT is severely impaired in the performance of processive DNA polymerization (16, 18, 20, 22). We have investigated the ability of the α-boranophosphonate diphosphates BH_3-PMEApp and BH_3-PMPApp to restore the processive DNA polymerization capability of R72A RT, such as in the case of α-boranophosphate analogues (7).

Determination of pre-steady-state kinetics is the method of choice to measure the substrate efficiency of a nucleotide analogue for RT. The efficiency of incorporation of a nucleotide into DNA is given by the ratio k_{pol}/K_d, k_{pol} being the rate constant for the creation of the phosphodiester bond and K_d being the equilibrium binding (or affinity) constant of the nucleotide for RT (15). Discrimination of an ANP relative to its natural counterpart deoxynucleoside triphosphate (dNTP) is reflected by the efficiency of incorporation of the ANP into DNA relative to that of the natural substrate. Although this has been done for various NRTIs, such constants have not been determined for BH_3-ANPs.

In this paper, we have made use of pre-steady-state kinetics to determine the affinity constant K_d as well as the catalytic constant k_{pol} of formation of the phosphodiester bond for dATP, PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp substrates by either WT or K65R RTs. Last, and as in the case of boranophosphate analogues, we observe improvement of catalysis due to the presence of the borane group in phosphonate analogues using R72A RT.

MATERIALS AND METHODS

Chemistry. All air-sensitive reactions were performed in oven-dried glassware under argon using extra-dry solvents from Aldrich. The ^1H, ^{13}C, and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AMX 250 (250-MHz) mass spectrometer, and the ^{11}B NMR spectrum was recorded with a Bruker AMX 400 mass spectrometer. Chemical shifts are expressed in ppm, and coupling constants (*J*) are in hertz (s, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; dt, double triplet; td, triple doublet; qd, quadruple doublet; m, multiplet; dm, double multiplet). Fast atom bombardment mass spectra (MS) and high-resolution MS (HRMS) were obtained on a JEOL SX 102 mass spectrometer using a cesium ion source and a glycerol-thioglycerol matrix. Analytical high-performance liquid chromatography (HPLC) analyses were carried out on a Waters Associates unit equipped with a model 600E multisolvent delivery system, a model 600E controller system, a model Rheodyne sample injector, a 991 photodiode array detector, and an in-line degasser AF. Samples were eluted using a linear gradient of 0.05 M triethylammonium bicarbonate buffer in 100% water (pH 7.5) (buffer A) to a 0.05 M triethylammonium bicarbonate buffer in 50% acetonitrile (buffer B), programmed over a 60-min period with a flow rate of 1 ml/min and detection at 260 nm. All solvents were of HPLC grade and filtered prior to use. A 1 M solution of triethylammonium bicarbonate buffer was prepared by adding dry ice to a 1 M triethylamine solution until the pH reached 7.5. Triethylammonium bicarbonate solutions were made fresh by dissolving reagent grade triethylammonium bicarbonate in HPLC grade water prior to filtration. Analytical reverse-phase chromatography was carried out on a 4.6- by 100-mm Source15RPC column. Preparative purifications of α-boranophosphonate derivatives were achieved on an ÄKTA*prime* fast-performance liquid chromatography instrument (Amersham) using a reverse-phase Source30RPC column (18 by 350 mm) and a linear gradient of buffer A to buffer B, programmed over a 6-h period with a flow rate of 2 ml/min and detection at 254 nm. Boranophosphonate nucleosides BH_3-PMEA and BH_3-PMPA were synthesized by following the procedure of Barral et al. (3). To prepare 5′-diphosphate analogues, Hoard and Ott's procedure (13) was adapted as follows. The boranophosphonates BH_3-PMEA and BH_3-PMPA were treated with an excess of 1,1′-carbonyldiimidazole (CDI) in dimethylformamide (DMF) and stirred for 4 h. The activated intermediates were not isolated. Unreacted CDI was decomposed with anhydrous methanol, before bis(tri-*n*-butylammonium) pyrophosphate was added. The phosphorylation was essentially complete within 72 h, and the products were purified by reversed-phase chromatography, followed by conversion to the sodium salt to conduce to BH_3-PMEApp and BH_3-PMPApp with 26 and 37% yields, respectively. Non-boranophosphonate diphosphates PMEApp and PMPApp, which serve as reference compounds, were synthesized as described in the literature (14).

(i) BH_3-PMEApp. BH_3-PMEA (18 mg, 0.066 mmol) was dissolved in DMF (2 ml) and treated with CDI (43 mg, 0.266 mmol). The resulting mixture was stirred at room temperature for 4 h. Excess CDI was decomposed by addition of anhydrous methanol (8 μl), and stirring was continued for 30 min. Anhydrous tri-*n*-butylamine (47 μl) and tributylammonium pyrophosphate (660 μl of a 0.5 M solution in DMF) were added, and the mixture was stirred at room temperature for 3 days. The reaction was stopped by the addition of 5 ml of cold water. The solvent was removed under vacuum, and the residue was purified by reversed-phase column chromatography (linear gradient of 0 to 100% buffer B). The appropriate fractions were collected, evaporated to dryness, and lyophilized. The residue was dissolved in water and passed through a Dowex 50WX2 (Na^+ form) column to give BH_3-PMEApp as trisodium salt (7.3 mg, 26%; HPLC purity, >99%); ^1H NMR (D_2O) δ: 7.95 (s, 2H, H-8, H-2), 4.15 (t, J = 4.5 Hz, 2H, CH_2N), 3.68 (t, J = 4.5 Hz, 2H, CH_2O), 2.94 (m, 2H, CH_2P), 0.5-(-) 0.10 (q, J = 90 Hz, 3H, BH_3); ^{31}P NMR (D_2O) δ: 104.09, −10.21, −20.90. HRMS (TOF, ES^-) was calculated for $C_8H_{16}N_5O_9P_3B$ $(M)^-$ as 430.0254 (found, 430.0267).

(ii) BH_3-PMPApp. BH_3-PMPA (22 mg, 0.077 mmol) was dissolved in DMF (2 ml) and treated with CDI (50 mg, 0.310 mmol). The resulting mixture was stirred at room temperature for 4 h. Excess CDI was decomposed by addition of anhydrous methanol (10 μl), and stirring was continued for 30 min. Anhydrous tri-*n*-butylamine (55 μl) and tributylammonium pyrophosphate (776 μl of a 0.5 M solution in DMF) were added, and the mixture was stirred at room temperature for 3 days. The reaction was stopped by the addition of 5 ml of cold water. The solvent was removed under vacuum, and the residue was purified by reversed-phase column chromatography (linear gradient, 0 to 100% buffer B). The appropriate fractions were collected, evaporated to dryness, and lyophilized. The residue was dissolved in water and passed through a Dowex 50WX2 (Na^+ form) column to give BH_3-PMPApp as trisodium salt (12.6 mg, 37%; HPLC purity, >98%); ^1H NMR (D_2O) δ: 7.96 (d, J = 9.5 Hz, 1H, H-8), 7.88 (d, J = 4.2 Hz, 1H, H-2), 4.06 (dd, J = 14.0 Hz and J = 2.8 Hz, 1H, CH_aN), 3.93 (m, 1H, CH_bN), 3.66 (m, 1H, CHO), 3.17 (m, 1H, CH_aP), 4.06 (dd, J = 13.0 Hz and J = 3.0 Hz, 1H, CH_bP), 0.84 (d, J = 6.3 Hz, CH_3), 0.2-(-) 0.80 (q, J = 93 Hz, 3H, BH_3); ^{31}P NMR (D_2O) δ: 99.37, −10.57, −21.19. HRMS (TOF, ES^-) was calculated for $C_9H_{18}N_5O_9P_3B$ $(M)^-$ as 444.0410 (found, 444.0392).

RT assays. (i) HIV RT plasmid constructions, enzyme preparations, and reagents. The WT RT bacterial expression gene construct p66RTB was used to obtain K65R and R72A RTs as described previously (5). All constructs were verified by DNA sequencing. The recombinant RTs were coexpressed with HIV-1 protease in *Escherichia coli* in order to obtain p66/p51 heterodimers, which were later purified using affinity chromatography. All enzymes were quantified by active-site titration before biochemical studies. DNA oligonucleotides were obtained from Life Technologies, United Kingdom. Oligonucleotides were 5′ ^{32}P labeled using T4 polynucleotide kinase (New England Biolabs, MA). [γ-^{32}P]ATP was purchased from Amersham Biosciences.

(ii) Drug susceptibility assays using recombinant HIV-1 RTs. Standard RT activity was assayed using 250 μg activated calf thymus DNA per ml. To determine 50% inhibitory concentration (IC_{50}) values for PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp, reactions were performed with 10 nM HIV-1 RT and 5 μM of each dNTP containing 100 μCi per mmol of [^3H]dATP, for 15 min with increasing amounts of inhibitor. Each aliquot was spotted in duplicate on DE81 ion-exchange paper disks, and the disks were washed three times for 10 min in 0.3 M ammonium formate, pH 8.0, and two times in ethanol and dried.

TABLE 1. Drug susceptibilities of WT RT for PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp

RT	Avg IC_{50} (μM) ± SD[a] for:			
	PMEApp	BH_3-PMEApp	PMPApp	BH_3-PMPApp
WT	8.3 ± 1.5	17 ± 1.8	22 ± 1	14.5 ± 1.2
K65R	52 ± 6 (6)	12 ± 1.6 (0.7)	170 ± 7 (8)	18 ± 1.5 (1.2)

[a] IC_{50} testing was conducted with recombinant RTs assayed on activated calf thymus DNA (see Materials and Methods) and averaged from at least three independent experiments. Values in parentheses are factors by which the K65R RT differs from WT RT.

The radioactivity bound to the filters was determined by liquid scintillation counting. Values of IC_{50} are the averages from at least three independent experiments.

(iii) Pre-steady-state kinetics of single nucleotide incorporation into DNA by RT. Pre-steady-state kinetics were determined using dATP, PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp, in conjunction with WT and K65R RTs. Rapid quench experiments were performed with a Kintek instrument, model RQF-3, using reaction times ranging from 10 ms to 30 s. All of the concentrations listed in this paragraph and the next are final. The primer DNA/DNA oligonucleotides used for the rapid reaction were a 5′-end-labeled 21-mer primer (5′-ATA CTT TAA CCA TAT GTA TCC-3′) annealed to 31-mer template 31A-RT (5′-AAA AAA AAA TGG ATA CAT ATG GTT AAA GTA T-3′). For natural nucleotides, the reaction was performed by mixing a solution containing 50 nM (active sites) of HIV-1 RT bound to 100 nM of primer/template in RT buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.05% Triton X-100) and a variable concentration of dNTP in 6 mM $MgCl_2$. Reactions involving ANPs were conducted with excess concentrations of enzyme (200 nM) over a primer/template duplex (100 nM). These conditions were chosen to eliminate the influence of the enzyme turnover rate (k_{ss}), which interferes in the measurements of low incorporation rates. Products of reactions were analyzed using sequencing gel electrophoresis (14% acrylamide and 8 M urea in Tris-borate-EDTA buffer) and quantified using photostimulated plates and FujiImager. The formation of product (P) over time was fitted with the burst equation $P = A \cdot [1 - \exp(-k_{app} \cdot t) + k_{ss} \cdot t]$, where A is the amplitude of the burst, k_{app} is the apparent kinetic constant of formation of the phosphodiester bond, and k_{ss} is the enzyme turnover rate, i.e., the kinetic constant of the steady-state linear phase. The dependence of k_{app} on dNTP concentration is described by the hyperbolic equation $k_{app} = k_{pol} \cdot [dNTP]/(K_d + [dNTP])$, where K_d and k_{pol} are the equilibrium constant and the catalytic rate constant of the dNTP for RT, respectively. K_d and k_{pol} were determined from curve fitting using Kaleidagraph (Synergy Software, PA).

(iv) DNA polymerization rate assays for RTs. The rate of polymerization was measured using a 5′-end-labeled oligo$(dA)_{21}$ annealed to a poly$(rU)_{31}$, in conjunction with WT and R72A RTs. Extension products were analyzed in a gel assay. The primer/template (25 nM) was incubated with 50 nM RT in RT buffer at 37°C. The reaction was initiated by the addition of 500 μM of nucleotide (dATP) or analogues (PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp) in 6 mM $MgCl_2$ and quenched at various times by 0.3 M EDTA. The percentages of elongated primer were determined from the product profile analysis using a FujiImager. Average polymerization rates were calculated for dATP by measuring the amount of the most abundant band product divided by time, to yield an apparent polymerization rate in nucleotides per second.

RESULTS

Enzyme susceptibility assays using steady-state kinetics. In order to examine the relative inhibitory capacity of ANPs, we tested the susceptibility of WT and K65R RTs to PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp. Results are summarized in Table 1. We observe that BH_3-PMEApp (IC_{50} = 17 μM) is a slightly less efficient inhibitor than PMEApp (IC_{50} = 8.3 μM), whereas BH_3-PMPApp (IC_{50} = 14.5 μM) is slightly better than PMPApp (IC_{50} = 22 μM). BH_3-PMEApp and BH_3-PMPApp are thus active in vitro against purified WT RT. As described before (25, 26), we observe that the K65R mutation induces a six- to eightfold resistance in vitro to both PMEApp (IC_{50} = 52 μM) and PMPApp (IC_{50} = 170 μM). Remarkably, however, susceptibility assays performed on K65R RT show that the potencies of both BH_3-PMEApp (IC_{50} = 12 μM) and BH_3-PMPApp (IC_{50} = 18 μM) are similar to that of WT RT, reversing apparent resistance. In the case of BH_3-PMEApp, K65R RT is 0.7-fold resistant (i.e., more sensitive) compared to WT RT. In the case of BH_3-PMPApp, K65R RT is not significantly resistant (1.2-fold) compared to WT. We conclude that boranophosphonate analogues are better inhibitors than their nonboranophosphanate counterparts on K65R RT.

Single incorporation of PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp into DNA by WT RT. Using pre-steady-state kinetics for single nucleotide incorporation into DNA, we measured the nucleotide initial binding affinity (K_d) and the subsequent burst rate of catalysis (k_{pol}). The ratio (k_{pol}/K_d) defines the nucleotide efficiency. For a given RT, comparing k_{pol}/K_d values for a natural dNTP and its corresponding analogue is a convenient way to assess selectivity for the natural nucleotide or discrimination of the analogue. For the sake of clarity, we will refer only to the discrimination—not the selectivity—of the analogue relative to its natural counterpart. Comparing discrimination levels between RTs defines in vitro resistance at the enzymatic level. The kinetic constants of dATP, PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp are reported in Table 2.

It is striking that BH_3-PMPApp is a better substrate than PMPApp. Indeed, discrimination relative to the natural dATP substrates decreases from 21.9- to 9.6-fold for PMPApp and BH_3-PMPApp, respectively. Perhaps the most apparent basis for this improvement of BH_3-PMPA is that it originates mainly from a more-than-threefold k_{pol} effect (from 7 to 23 s^{-1}). This is not the case for PMEApp and BH_3-PMEApp, and this parallels steady-state data satisfactorily (see above).

Single incorporation of PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp into DNA by K65R RT. Assays of the same type were performed in the case of the resistant K65R variant of RT. Strikingly, in this case, both PMEApp and PMPApp discriminations are decreased in the presence of the BH_3 group (17.9- to 8.8-fold and 97.8- to 10-fold, respectively). As a consequence, resistance is also suppressed in both cases, and a 2.5-fold hypersensitivity (or 0.4-fold resistance) is even observed in the case of BH_3-PMEApp. In the same vein as for WT RT, the presence of the α-borane group has a larger effect on k_{pol} than K_d. In the case of PMPApp, the presence of the BH_3 group results in a 5.8/0.32 (18)-fold increase in k_{pol}. In conclusion, compounds BH_3-PMEApp and BH_3-PMPApp both inhibit K65R RT efficiently and are better inhibitors than their respective PMEApp and PMPApp counterparts.

Comparison between WT and K65R RTs for PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp. Comparing discrimination levels between RTs defines in vitro resistance at the enzymatic level. Expectedly, as shown in Table 2, the K65R RT variant is resistant to PMEApp and PMPApp (2.3- and 4.5-fold, respectively). The resistance is due to a decrease of the catalytic rate constant, as described before (21). The increased discrimination of PMPApp compared to dATP brought by the K65R mutation accounts for the phenotypic loss of susceptibility of the mutated virus to the drug in infected cell cultures (9, 25, 26). But in the presence of the α-borane analogue, the resistance of K65R RT is decreased to

TABLE 2. Pre-steady-state kinetic constants of dATP, PMEApp, PMPApp, BH3-PMEApp, and BH_3-PMPApp and incorporation by HIV-1 RTs[a]

RT	Nucleotide or nucleotide analogue	k_{pol} (s^{-1})	K_d (μM)	k_{pol}/K_d ($s^{-1} \cdot \mu M^{-1}$)	Discrimination[c]	Resistance[d]
WT	dATP	50	7.5	6.7		
	PMEApp	6.8	7.9	0.9	7.7	
	BH_3-PMEApp	2	6.5	0.3	21.7	
	PMPApp	7[b]	23[b]	0.3	21.9	
	BH_3-PMPA	23	33	0.7	9.6	
K65R	dATP	12	6.9	1.7		
	PMEApp	0.75	7.7	0.1	17.9	2.3
	BH_3-PMEApp	1.4	7.1	0.2	8.8	0.4
	PMPApp	0.32[b]	18[b]	0.02	97.8	4.5
	BH_3-PMPApp	5.8	35	0.17	10	1

[a] K_d and k_{pol} were determined as described in Materials and Methods. Standard deviations were <20% for three independent experiments.
[b] Value from Deval et al. (9).
[c] Ratio of k_{pol}/K_d for the nucleotide to k_{pol}/K_d for the nucleotide analogue.
[d] Ratio of discrimination for the mutant to discrimination for the WT.

1-fold (i.e., nonexistent) for BH_3-PMPApp and to 0.4-fold (i.e., hypersensitivity) for BH_3-PMEApp. This decrease of resistance results from 18- and 1.9-fold increases in k_{pol}, respectively. We conclude that, remarkably, the presence of the α-borane group renders K65R RT hypersensitive to the BH_3-PMEApp analogue. In the case of BH_3-PMPApp, the presence of α-borane suppresses the K65R RT-mediated resistance observed with PMPApp.

Polymerase activity of the active-site mutant R72A RT with ANPs and BH_3-ANPs. A polymerization rate assay was used in conjunction with WT or R72A RT to measure the incorporation rate of dATP, PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp along a poly$(rU)_{31}$ RNA template during the early polymerization events (Fig. 2). On average, we observe that WT RT incorporates dATP at a 4.6-fold-higher rate than R72A RT, i.e., 0.58 nucleotide (nt) $\cdot$ s^{-1} and 0.125 nt $\cdot$ s^{-1} for WT and R72A, respectively, as described before (20). Product profile analysis shows that these values represent 75 and 23%, respectively, of that for the elongated primer for a reaction time of 10 seconds (lanes 9 and 19, respectively). Under these same experimental conditions, PMEApp and PMPApp chain-terminated DNA products produced by WT RT represent 72% and 74% (lanes 1 and 3, respectively). These product yields are similar to those observed when the α-borane group is present on the α-phosphates of BH_3-PMEApp and BH_3-PMPApp (69 and 74%, respectively [lanes 5 and 7, respectively]). Comparatively, R72A RT incorporates PMEApp and PMPApp very poorly (1.6 and 0.2%, respectively [lanes 11 and 13, respectively]), but these low polymerization rates are improved dramatically by using their α-borane analogues BH_3-PMEApp and BH_3-PMPApp (53 and 45%, respectively [lanes 15 and 17, respectively]). We conclude that the α-borane group indeed

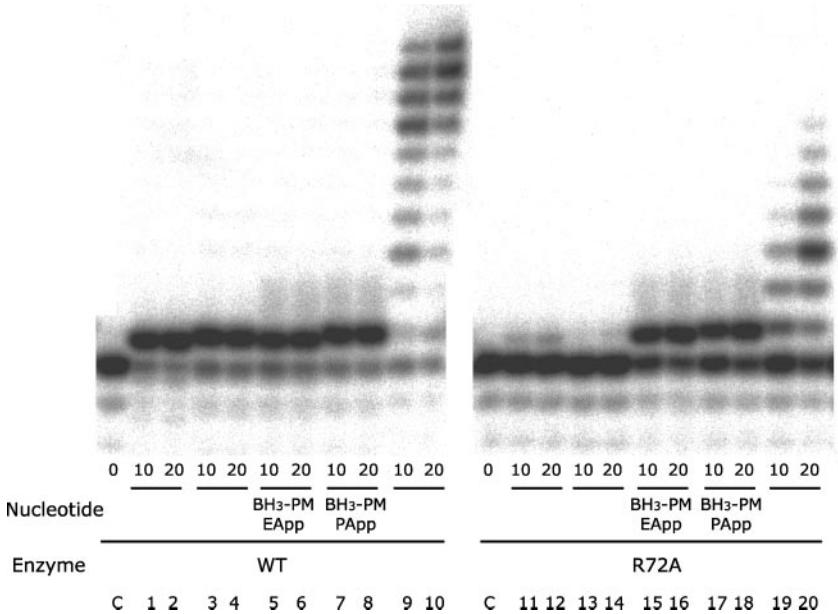

FIG. 2. Polymerization rate assay using dATP, PMEApp, PMPApp, BH_3-PMEApp, and BH_3-PMPApp. WT and R72A RTs were assayed in the first seconds of the primer extension reaction on a primer oligo$(dA)_{21}$ annealed to a template poly$(rU)_{31}$ with dATP or analogues. Shown is an autoradiograph of the reaction products separated using denaturing gel electrophoresis (see Materials and Methods).

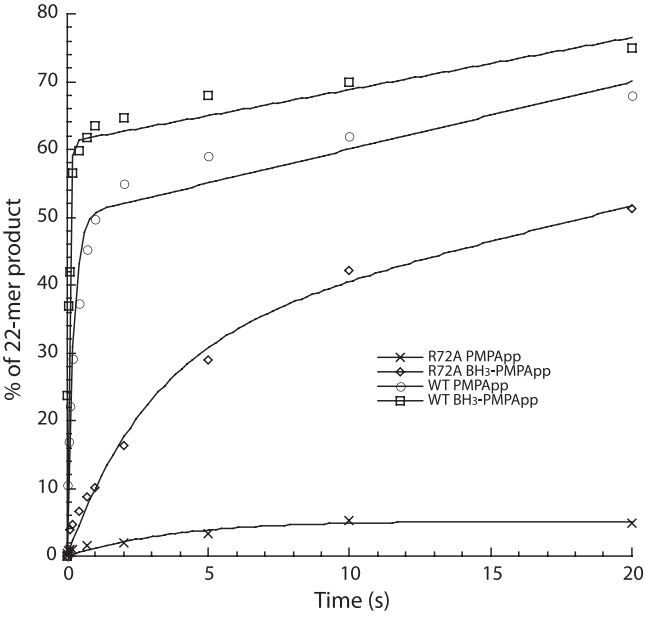

FIG. 3. Kinetics of a single nucleotide incorporation into DNA using PMPApp and BH_3-PMPApp in conjunction with WT and R72A RTs. The latter were assayed in the first seconds of the primer extension reaction on a 21-mer primer annealed to a 31-mer template, allowing incorporation of the adenine nucleotide analogue into a 22-mer product (see Materials and Methods).

stimulates catalysis. It provides the nucleotide with intrinsic chemical properties able to circumvent the absence of the key catalytic amino acid R72, as described before for α-boranophosphate nucleotides (7).

Single nucleotide incorporation of PMPApp and BH_3-PMPApp into DNA by WT and R72A RTs using a single 500 μM concentration of analogue. The study of the restoration of the catalytic rate constant upon the presence of a borane group was refined in the case of compounds PMPApp and BH_3-PMPApp using pre-steady-state kinetics. As shown in Fig. 3, the apparent kinetic constant of formation of the phosphodiester bond (k_{app}) increases 3.4-fold for the BH_3-PMPApp borane analogue (16 s^{-1}) compared to PMPApp using WT RT (4.7 s^{-1}). The effect is even greater in the case of R72A RT, where k_{app} for BH_3-PMPApp (0.33 s^{-1}) increases more than 10-fold compared to that for PMPApp (0.03 s^{-1}). We conclude that, seemingly similar to what was found for α-boranophosphate nucleosides, the presence of the α-borane group increases the incorporation efficiency in WT RT and restores the catalytic properties of R72A RT.

DISCUSSION

We describe here the synthesis of the first α-boranophosphonate diphosphate nucleosides in which a borane (BH_3^-) group replaces one nonbridging oxygen atom of the α-phosphonate moiety. Compounds BH_3-PMEA and BH_3-PMPA previously synthesized are inactive against HIV-1 in infected cell cultures, however, due to their rapid degradation in an extracellular medium mimic (3). This suggests that chemical modification ("vectorization") should be considered to prevent compound catabolism and promote cell penetration. These BH_3-PMEApp and BH_3-PMPApp analogues could then potentially become potent resistance suppressors useful in the clinic.

The compounds described here present interesting properties, however. Assays of the enzyme susceptibility of BH_3-PMEApp and BH_3-PMPApp on HIV-1 RTs show that these compounds inhibit WT RT in the same range as that of PMEApp and PMPApp. Furthermore, boranophosphonate analogues are better inhibitors than their unmodified PMEApp and PMPApp counterparts using K65R RT. Pre-steady-state kinetics of single nucleotide incorporation of BH_3-PMEApp and BH_3-PMPApp on HIV-1 RT allow a closer look on the reaction. The presence of the α-borane group has a beneficial effect on k_{pol}, albeit in the case of BH_3-PMPApp only.

HIV-1 strains encoding a K65R mutant RT are selected in vivo by tenofovir, and K65R seems to reduce the incorporation of tenofovir into DNA (26). K65R HIV-1 RT has a reduced pre-steady-state rate of dNTP incorporation (21), suggesting that the reduced incorporation of tenofovir comes at a cost to incorporation of natural dNTP. Studies on K65R RT reveal that compounds BH_3-PMEApp and BH_3-PMPApp inhibit K65R RT efficiently and are better inhibitors than PMEApp and PMPApp, respectively. Therefore, K65R RT is not more resistant to BH_3-PMPApp than to PMPApp and is hypersensitive to BH_3-PMEApp compared to PMEApp. The presence of the borane group does not influence the binding of the analogue to the RT active site but provides (or restores) a high k_{pol} of incorporation of the analogue specifically, as described previously for boranophosphates (1).

The active-site mutant R72A RT is severely impaired in its ability to perform processive DNA polymerization. The R72A RT shows a defect in eliminating the pyrophosphate produced at the active site upon incorporation of the nucleotide into DNA (16, 18, 20, 22). However, the presence of the borane group on the α-phosphate of 2′-deoxynucleotides restores the processive DNA polymerization capability of R72A RT (7). It was of interest to verify if this property would also be observed using α-boranophosphonate diphosphates BH_3-PMEApp and BH_3-PMPApp. WT RT incorporates BH_3-PMEApp and BH_3-PMPApp in the same manner as non-boranophosphonate diphosphates. Comparatively, R72A RT incorporates PMEApp and PMPApp very poorly, but these low polymerization rates are improved dramatically using their α-borane analogues BH_3-PMEApp and BH_3-PMPApp. More precisely, the k_{app} with RT is improved for the BH_3-PMPApp borane analogue compared to PMPApp. This improvement is even greater in the case of R72A RT, where the difference between k_{app} values for BH_3-PMPApp and PMPApp is increased. The presence of the α-borane group increases the catalytic rate of WT RT and restores the catalytic properties of R72A RT. The borane modification provides the nucleotide with intrinsic chemical properties able to circumvent the presence of the key catalytic amino acid R72, as described before for α-boranophosphate nucleotides (7), and shows a capability to compensate for a mutation inducing a defect in the nucleotide incorporation rate.

In conclusion, these novel nucleotide analogues demonstrate not only that the α-phosphate of phosphonate analogues can be modified without jeopardizing incorporation properties but also that this modification could be useful to circumvent

HIV RT-mediated drug resistance. It would be interesting to assess the use of other α-phosphonate modifications, such as thio- or selenophosphonates, not only for their incorporation efficiency but also to improve the stability of analogue-terminated DNA chains.

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